CCQM-P55.2.c

Pilot Study on Peptide Purity - Glycated Hexapeptide of HbA1c

Final Report

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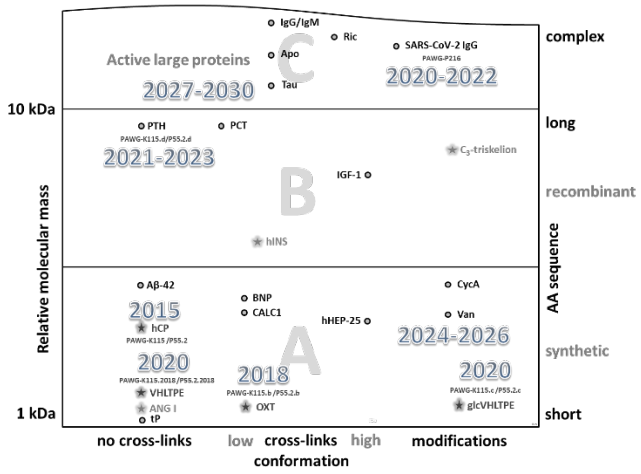
INTRODUCTION

Comparability of (bio)chemical measurements is a prerequisite of any measurement undertaken in support of legislative purposes. For most chemical analysis this can be achieved by ensuring that measurement results are traceable to a known reference such as the base units of the Système International d'Unités (SI) [1]. By maintaining such a link, results can be compared over time and space enabling informed decisions to be made and improving our overall knowledge of a subject area. The importance of traceable measurement results can be inferred by its requirement in quality standards (ISO 17025) and in the formation of specialized committees as the Joint Committee on Traceability in Laboratory Medicine (JCTLM). However, whilst the required metrological tools, such as higher order reference measurements procedures, pure substance and matrix certified reference materials, are established for small well defined molecules difficulties still remain in the provision of such standards in the area of larger biomolecules notably peptides/proteins.

The provision of Primary Calibration Reference Services has been identified as a core technical competency for National Measurement Institutes (NMIs) and Designated Institutes (DIs) [2]. NMIs/DIs providing measurement services in peptide/protein analysis are expected to participate in a limited number of comparisons that are intended to test and demonstrate their capabilities in this area.

Primary Calibration Reference Services refers to a technical capability for composition assignment, usually as the mass fraction content, of a peptide/protein in the form of high purity solids or standard solutions thereof.

The assignment of the mass fraction content of high purity materials is the subject of the CCQM-K115 comparison series. A model to classify peptides in terms of their, relative molecular mass, the amount of cross-linking, and modifications has been developed and upgraded as it is depicted in Figure 1 [1,3]. With the aim of leveraging the work required for the CCQM-K115 comparison and thereby minimising the workload for NMIs/DIs and simultaneously focussing on a material directly relevant to existing CMC claims, human C-peptide (hCP) was the most appropriate choice for a study material for a first CCQM key comparison and parallel pilot study looking at competencies to perform peptide purity mass fraction assignment. hCP covers the space of quadrant A of the model as it allowed generic capabilities to be demonstrated for linear peptides without cross-links and of up to 31 amino acids in length [4,5]. The second cycle of peptide purity comparisons, CCQM-K115.b/P55.2.b on oxytocin (OXT) covered the space of quadrant A for short (1 kDa to 5 kDa), cross-linked and non-modified synthetic peptides as OXT is a cyclic peptide possessing nine amino acid residues and a disulfide bond. OXT is a chemically synthesized peptide hormone [6,7].



Peptide	AAs	kDa	SSs
Hexapeptide of HbA0 (VHLTPE or VE)	6	~0.7	0
Glycated hexapeptide of HbA1c (glcVHLTPE or GE)	6	~0.8	0
Tryptic Peptide Solution (tP)	~10	~1	0
Oxytocin (OXT)	9	1	1
Angiotensin I (ANG I)	10	1.2	0
Human Hepcidin-25 (hHEP-25)	25	2.8	4
Human C-Peptide (hCP)	31	3	0
Calcitonin Salmon (CALC1)	32	3.4	1
Brain Natriuretic Peptide (BNP)	32	3.5	1
Amyloid-Beta-42 (Aβ-42)	42	4.5	0
Human Insulin (hINS)	51	5.8	3
Insulin-like growth factor 1 (IGF-1)	70	7.6	3
C_3-triskelion (antimicrobial peptide capsid)	74	8.3	0
Parathyroid hormone (PTH)	84	9.4	0

Figure 1: Model for the classification of peptides for primary structure purity determinations

RATIONALE/PURPOSE

The approach taken for small molecules relies on Primary Calibrators, often in the form of a synthetic standard of known purity. The provision of Primary Calibration Reference Services has been identified as a core technical competency for NMIs/DIs in the strategy developed for the planning of ongoing Key Comparisons of the Organic Analysis Working Group (OAWG) within the Comité Consultatif pour la Quantité de Matière (CCQM) [8]. NMIs/DIs providing measurement services in organic analysis are expected to participate in a limited number of Track A comparisons that are intended to test and demonstrate their capabilities in this area. Primary Calibration Reference Services refers to a technical capability for composition assignment, usually as the mass fraction content, of organic compound(s) such as pure substances or solutions. The procedure adopted by most NMIs/DIs, for the provision of primary pure substance calibrators relies on a mass balance approach. This can be determined either by approaches that measure the mass fraction or mole fraction of the main component directly, or by indirect approaches that identify and estimate the mass fraction of the individual impurities and/or distinct classes of impurities present in the material and, by subtraction, provide a measure for the main component of the material [9]. These approaches have been successfully applied to a large variety of small molecules [10-14].

The quantification of larger molecules is complicated by the fact that they can exhibit higher order structures, and that characterization of the primary structure of the molecule maybe insufficient to correlate the amount of the molecule to its biological activity. Nevertheless, the quantification of the primary structure purity of a larger molecule is the first step in establishing a primary calibrator material for that molecule, where the quantity of interest is the mass fraction of the large molecule. The current discussion is limited to the measurement of the primary structure mass fraction of the molecule within a material.

Another complication for the provision of traceable peptide/protein measurements is that pure peptides/proteins can usually not be obtained in sufficiently large quantities. This has resulted in the harmonisation of many large molecule measurements by the provision of accepted practices, methods and/or standards. However, the increased use of targeted hydrolysis based digestion and peptide quantification strategies has enabled the determination of protein amounts via prototypic peptides [15-17]. These approaches have been investigated for example for the routine analysis of human growth hormone and its biomarkers [18-19]. A number of NMIs/DIs have been developing higher order measurement procedures for the analysis of purified protein calibrators [20] and serum based matrix materials [19]. These approaches show great promise for the standardisation of priority protein measurands. However, the mass fractions value assignment of proteins requires proteotypic peptides of known purity [1].

The purity of proteotypic peptides and peptides that show direct bioactivity by themselves can be assessed by use of the full mass balance approach. However, a full mass balance approach could require unviably large quantities of peptide material. A simpler alternative to the full mass balance approach is a peptide impurity corrected amino acid (PICAA) analysis, requiring quantification of constituent amino acids following hydrolysis of the material and correction for amino acids originating from impurities [4-7, 21-22]. It requires identification and quantification of peptide impurities for the most accurate results.

Traceability of the amino acid analysis results is to pure amino acid certified reference materials (CRMs). Few pure amino acid CRMs are commercially available. Alternatively, traceability could be established through in-house or NMI purity capabilities for amino acids. NMI capabilities to determine the purity of L-valine, were assessed in the CCQM-K55.c comparison in the frame of the OAWG [12]. In addition, amino acid analysis and peptide hydrolysis capabilities for the mass concentration assignment of peptide solutions are evaluated in the series of CCQM-P55 comparisons in the framework of the former BAWG using peptide materials of unknown purity [1].

The application of other approaches for the assessment of peptide purity that require only minor quantities of peptide material is conceivable, for example elemental analysis (CHN/O) with a correction for nitrogen originating from impurities or quantitative nuclear magnetic resonance (qNMR) spectroscopy with a correction for structurally-related peptide impurities (PICqNMR) [1, 4, 23].

The timeline for the CCQM-P55.2.c study ‘Pilot Study on Peptide Purity - Glycated Hexapeptide of HbA1c’ parallel to CCQM-K115.c is summarized in Table 1.

Table 1: CCQM-P55.2.c Timetable

Action	Date
Initial discussion	October 2016 and April 2017 PAWG meetings
Approval of Study Proposal	September 2017 PAWG meeting
Draft protocol and confirmation	April 2018 PAWG meeting
Sample characterization completed	January 2019
Call for participation	April 1st, 2019
Final date to register	April 30th, 2019
Sample distribution	June to July 2019
Date due to coordinator	September 18th, 2020
Justification for 14 months period	Shifted several times because of the coronavirus pandemic
Initial report and discussion of results	November 2020 PAWG meeting
Discussion and reference value established	April 2021 PAWG meeting
Draft B report	March 2022 approved by PAWG
Final report to PAWG Chair	April 2022

CHARACTERIZATION OF STUDY MATERIAL

The mass fraction of the glycated hexapeptide of HbA1c (GE) in the material is to be determined. GE is defined as glycated hemoglobin subunit beta [2-7] fragment with the amino acid sequence glcVHLTPE and a relative molecular mass (M_r) of about 856.6 g/mol. The N-terminus valine of the hemoglobin β-chain has been converted to a stable adduct of glucose (1-deoxyfructosyl).
The study material was prepared by the BIPM/HSA by characterization of a commercially sourced sample of synthetic GE. The methods used to investigate, assign and confirm the quantitative composition of the CCQM-K115.c and CCQM-P55.2.c candidate material by the BIPM are summarized below.

CHARACTERIZATION STUDIES

Peptide related impurity content was evaluated by

- LC-hrMS/MS

Water content was evaluated by

- Coulometric Karl Fischer titration (KFT) with oven transfer of water from the sample
- Thermogravimetric analysis (TGA) as a consistency check for the assigned value
- Microanalysis (% C, H, N content) as a consistency check for assigned value
- Sorption balance measurements

Residual solvent content was evaluated by

- GC-MS by direct injection
- ^{1}H-NMR
- Thermogravimetric analysis as a consistency check for the assigned value
- Microanalysis (% C, H, N content) as a consistency check for the assigned value

Non-volatile organic/ inorganic content by

- ^{19}F-NMR
- IC for common elements and counter ions (acetate, chloride, formate, nitrate, oxalate, phosphate, sulfate, trifluoroacetate (TFA), ammonium, calcium, magnesium, potassium, sodium) as a consistency check for the assigned values
- Microanalysis (% C, H, N content) as a consistency check for the assigned values

The BIPM/HSA have

- investigated the levels of within and between vial homogeneity of the main component and selected significant minor components;
- identified a minimum sample size which reduces to an acceptable level the effect of between-bottle inhomogeneity of both the main component and the minor components;
- completed isochronous stability studies of both the main component and the minor components to confirm that the material is sufficiently stable within the proposed time scale of the study if stored at low temperature (4 °C to - 20 °C);
- determined appropriate conditions for its storage (4 °C to - 20 °C), transport (cooled and temperature controlled) and handling;
- studied the impact of the relative humidity and temperature on the water content and provide a correction function for the gravimetric preparation of the comparison sample.

HOMOGENEITY AND STABILITY STUDIES AND SORPTION MEASUREMENTS

The batch of CCQM-K115.c and CCQM-P55.2.c candidate material vials were evaluated for impurity profile, homogeneity, stability and water adsorption/desorption by the BIPM/HSA. The mass fraction of the GE in the comparison material was assessed by the BIPM to be about 630 mg/g while the homogeneity and stability of the GE and peptide related impurities were shown to be suitable for the purpose of the comparison. Dynamic vapor sorption balance measurements indicated that weighings of the CCQM-K115.c and CCQM-P55.2.c comparison material need to be performed under controlled conditions of temperature and relative humidity (RH) as the water content of the comparison material changes reversibly as a function of the RH. A full summary of the results for GE mass fraction and of the methods used to investigate, assign and confirm the composition of the CCQMK115.c and CCQM-P55.2.c candidate material and to demonstrate the fitness for purpose of the homogeneity, stability and reversible water adsorption/desorption of the material are given in detail in the CCQM-K115.c Final Report [24].

SAMPLE DISTRIBUTION

Samples were distributed by HSA to all participants and the co-coordinating institutes (BIPM and NIM) in June and July 2019.
Two units of the study sample, each containing a minimum of 25 mg of materials, were distributed to each CCQM-P55.2.c participant by express mail service in insulated and cooled transport containers equipped with a temperature data logger to record the temperature throughout the transport process. Participants were asked to return the sample receipt form and the data logger report acknowledging receipt of the samples and to advise the coordinators if any obvious damage had occurred during the shipping. All CCQM-P55.2.c participants received the samples within one week from the time the samples were shipped out. The data logger reports to all participants except TUBITAK UME showed that the samples had not been exposed to temperature above 2 - 8 °C during the transport process. The data logger reports to TUBITAK UME showed that the samples had been exposed to temperature above 2 - 8 °C for about one day (highest temperature reached: 15.3 °C). As the time above 2 - 8 °C was very short and the temperature did not even reach room temperature, the coordinators concluded that the samples were still appropriate for study. It should be noted that CENAM, Mexico has also been supplied with samples to participate in CCQM-P55.2.c. However, CENAM was not able to finalize the study and submit results because of constraints due to the Coronavirus pandemic.

QUANTITIES AND UNITS

Participants were required to report the mass fraction of GE, the major component of the comparison sample. In addition, all participants who used a PICAA or qNMR procedure to determine the GE mass fraction were required to report the combined mass fraction assignment and corresponding uncertainty for total related peptide impurities.
In addition, the BIPM, HSA and NIM who employed a mass balance (summation of impurities) procedure to determine the GE mass fraction were required to report the combined mass fraction assignment and corresponding uncertainty for the sub-classes of total related peptide impurities, water, total residual organic solvent / volatile organic compounds (VOCs) and total non-volatile organics & inorganics. Details are provided in the CCQM-K115.c Final Report [24].
Participants were encouraged to also provide mass fraction estimates for the main impurity components they identified in the comparison sample.

REPORTED MASS FRACTIONS OF GE AND IMPURITIES IN CCQM-P55.2.C

The values reported by participants for the GE mass fraction in CCQM-P55.2.c are given in Table 2 with a summary plot in Figure 2. The values reported by participants for the peptide related impurity (PepImp) mass fractions in CCQM-P55.2.c are given in Table 3 with a summary plot in Figure 3.
The reported values for the GE mass fractions in CCQM-P55.2.c can be divided into two groups. Four participants have employed PICAA approaches and two participants have used PICqNMR approaches.

Table 2: Results for CCQM-P55.2.c: GE mass fractions and uncertainties as received

Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	GE	u(GE)	U(GE)		
NIM, China	667.76	13.20	26.40	2	PICAA
NIM, China	657.13	12.05	24.11	2	PICAA 2
BIPM	675.8	12.5	25.0	2	PICAA
BIPM	650.2	5.0	10.1	2	PICqNMR
UME, Turkey	600.7	4.7	9.4	2	PICqNMR
KRISS, Korea (Rep. of)	660.63	12.36	28.49	2.306	PICAA

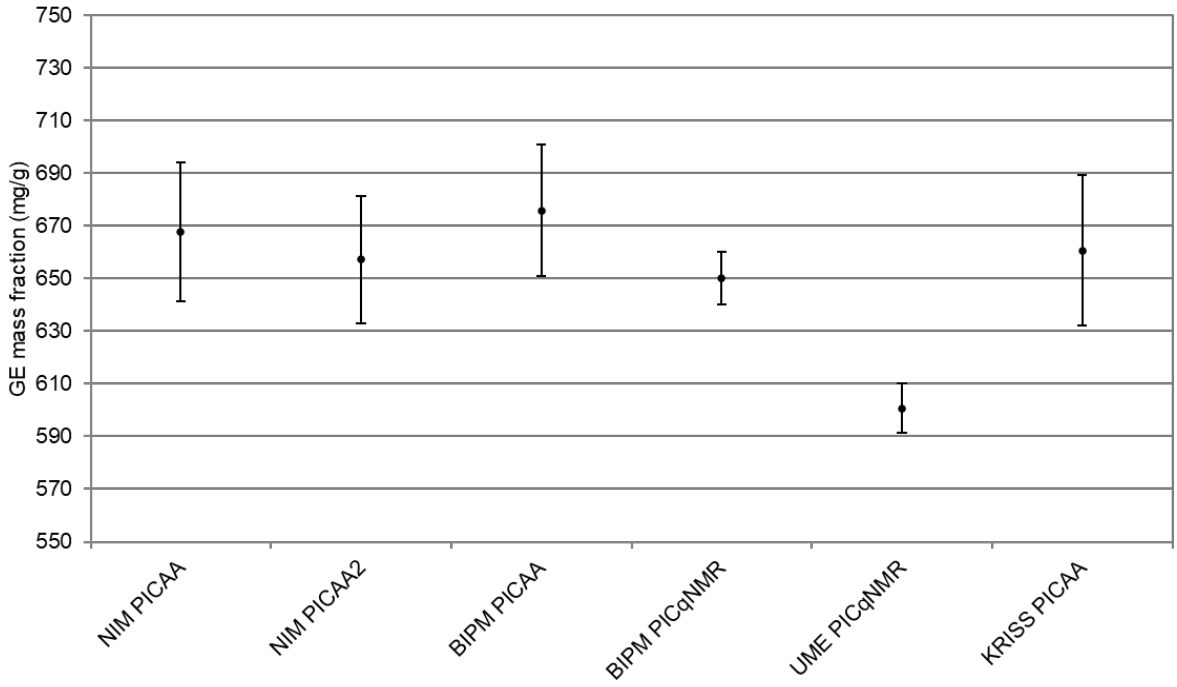


Figure 2: GE mass fractions reported by participants in CCQM-P55.2.c - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

Table 3: Results for CCQM-P55.2.c: Overall peptide related impurities (PepImp) mass fractions and uncertainties as received

Participant	Mass fractions (mg/g)			Coverage Factor (*k*)	Approach
	PepImp	*u*(PepImp)	*U*(PepImp)		
NIM, China	40.25	2.10	4.21	2	HPLC-MS/MS
NIM, China	40.25	2.10	4.21	2	HPLC-MS/MS
BIPM	36.5	1.8	3.6	2	LC-hrMS
BIPM	36.5	1.8	3.6	2	LC-hrMS
UME, Turkey	48.5	1.8	3.5	2	LC-hrMS
KRISS, Korea (Rep. of)	41.02	0.77	1.77	2.306	nanoLC-MS/MS

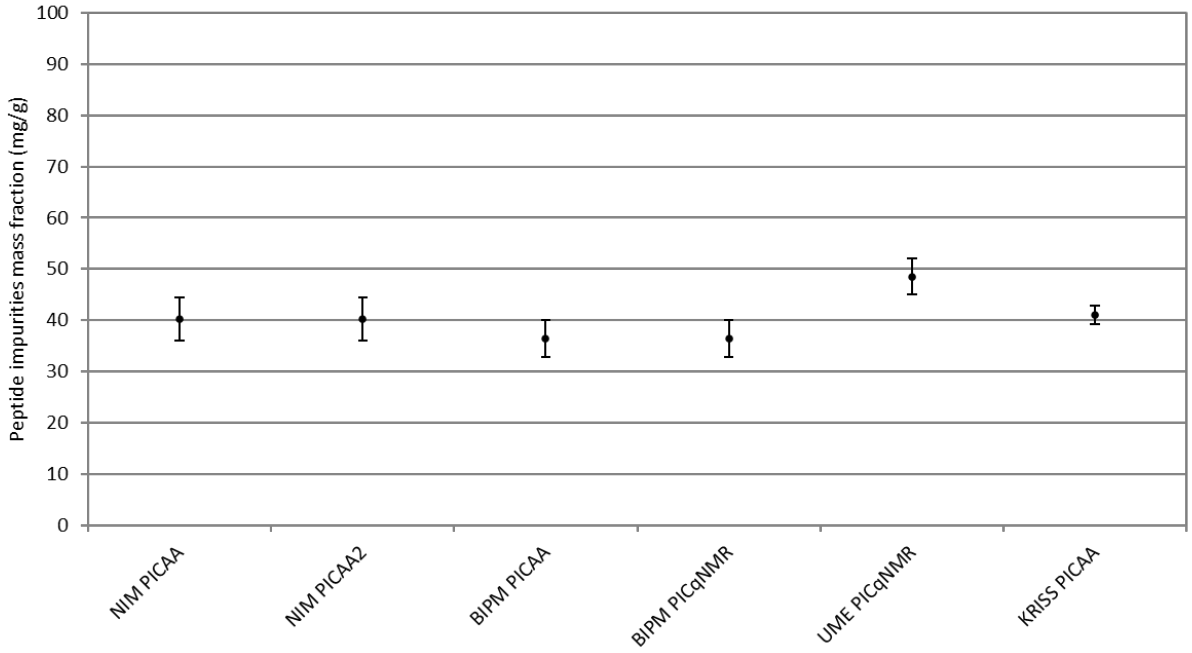


Figure 3: Overall peptide related impurities (PepImp) mass fractions reported by participants in CCQM-P55.2.c - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

In general, the CCQM-K115.c/P55.2.c comparison on GE purity shows less agreement of participants' results as the previous CCQM-K115/P55.2 series comparisons on hCP and OXT for peptide purity determinations. The peptide related impurity (PepImp) determinations showed the same level of agreement as for OXT. However, there was discussion on possible reasons for the discrepancy between CCQM-K115.c/P55.2.c results after presentation of the results of participants at the PAWG meeting in November 2020 and April 2021.

The peptide related impurities identification and quantification (Figure 4) is still a weak point as for both comparison on hCP and OXT as described in detail in the CCQM-K115.c Final Report [24]. The number of potential impurities is much smaller for GE compared with both hCP and OXT as GE exhibits a shorter primary sequence. All participants have identified/quantified the dominating major peptide related impurity VE resulting in mainly coherent estimations of the peptide related impurity mass fractions. However, the second largest peptide impurity, GE depsipeptide, has only been correctly identified and quantified by the NRC as described in detail in the CCQM-K115.c Final Report [24]. Hence most of the pilot study participants have slightly underestimated the sum of peptide related impurity mass fractions. A few key comparison participants, for example BIPM, LGC, HSA and LNE, have observed an additional broad peak but it was not identified as GE depsipeptide. It has been discussed if that peak could relate to the GE depsipeptide if certain solvent conditions are maintained in LC-MS analysis as the GE depsipeptide is only stable at low pH conditions for a few days. The depsipeptide issue is discussed in detail in the section Peptide Related Impurity Profile of CCQM-K115.c [24].

It has been pointed out that the use of synthesized impurity standards has a positive impact on the quantification of the peptide related impurity mass fractions. Four laboratories have used synthesized impurity standards to quantify the major impurity VE. Two participants have quantified the peptide related impurities using a response factor (RF = 1) method. NIM used 13 synthesized impurity standards (purities taken into account), BIPM used 4 synthesized impurities standards (purities taken into account) to quantify the individual impurities and closely structurally related impurities.
The BIPM and UME have used the PICqNMR approach in CCQM-P55.2.c. All other participants have used the PICAA approach. NIM has employed two different mass spectrometers for IDMS (NIM PICAA and NIM PICAA2). BIPM has used microwave assisted hydrolysis. KRISS and NIM have employed gas/liquid phase hydrolysis. However, all participants that have used PICAA have performed an efficiency correction for the hydrolysis methods. The peptide related impurities values have been broken down to establish a means to visualize identification and quantification issues for the peptide related impurities.

Peptide Related Impurity Profile of CCQM-K115.c

The BIPM has broken down the peptide related impurities values to establish a means to visualize identification and quantification issues for the peptide related impurities. Figure 4 shows more details on the peptide related impurities of the CCQM-K115.c or -P55.2.c studies. The graph shows the peptide impurities that have been identified, the mean of the corresponding mass fractions, the corresponding standard deviations and the corresponding number of laboratories that have identified and quantified that impurity. The maximum possible number of identifications is ten as there are ten theoretical independent data sets due to the fact that some laboratories have used the same peptide impurity data set twice for example to correct both PICAA and qNMR results.
Please note that several laboratories have identified groups of impurities but the position of the modification was not or not entirely identified, for example GE dimer isomers and GE(OMe). In the graph it has been considered as identified but the mass fraction value has not been used for the calculation of the means of peptide impurity mass fractions.
In general, the identification and quantification of peptide impurities is quite coherent among laboratories. However, certain issues were discussed during the PAWG meetings in November 2020 and April 2021.
The dominating major peptide related impurity VE has been identified and quantified by all ten laboratories. The sum of peptide related impurity mass fractions consists of about 75 % of VE. However, the second largest peptide impurity, GE depsipeptide, has only been correctly identified and quantified by the NRC via ^{1}H-NMR. The identification and quantification of the GE depsipeptide by use of LC-(hr)MS(/MS) techniques have proved to be difficult. Initially, the GE depsipeptide impurity was missed or misinterpreted by all participants using by LC-(hr)MS(/MS). It was confirmed that the GE depsipetide is only present in freshly prepared aqueous solution of the GE material. Aqueous solutions are acidic (about pH 4) due to the high TFA content of the GE

material. The GE depsipeptide peak decreased and disappeared completely after a few days (< 4 days) when the GE sample is prepared in an acidic aqueous solution (pH 4). The GE depsipeptide peak disappeared instantly when GE materials were dissolved in alkaline buffer (pH 9). In addition, HSA proved, for the example of VE depsipeptide, that the depsipeptide transformation in alkaline or weak acid solution (pH > 4) is irreversible (no depsipeptide production upon re-acidification to pH ~ 2.5). These findings imply that the GE depsipeptide was already present in the solid material. It should be noted that the instability of depsipeptide impurities could impact measurements for clinical purposes if the LC-MS methods used are employed under alkaline conditions.

Furthermore, it has been decided during the discussions within the CCQM PAWG in April 2021 that the GE depsipeptide structural isomer would be counted as impurity whereas the stereoisomers *cis/trans*, also present in the material, would not by counted as separate impurities.

UME has also re-assessed their data and in retrospect reported an identification mismatch of Glc-AVHLTPEE to Glc-VHLTAPE iso A. Details on the GE depsipeptide issue are provided in the CCQM-K115.c Final Report [24].

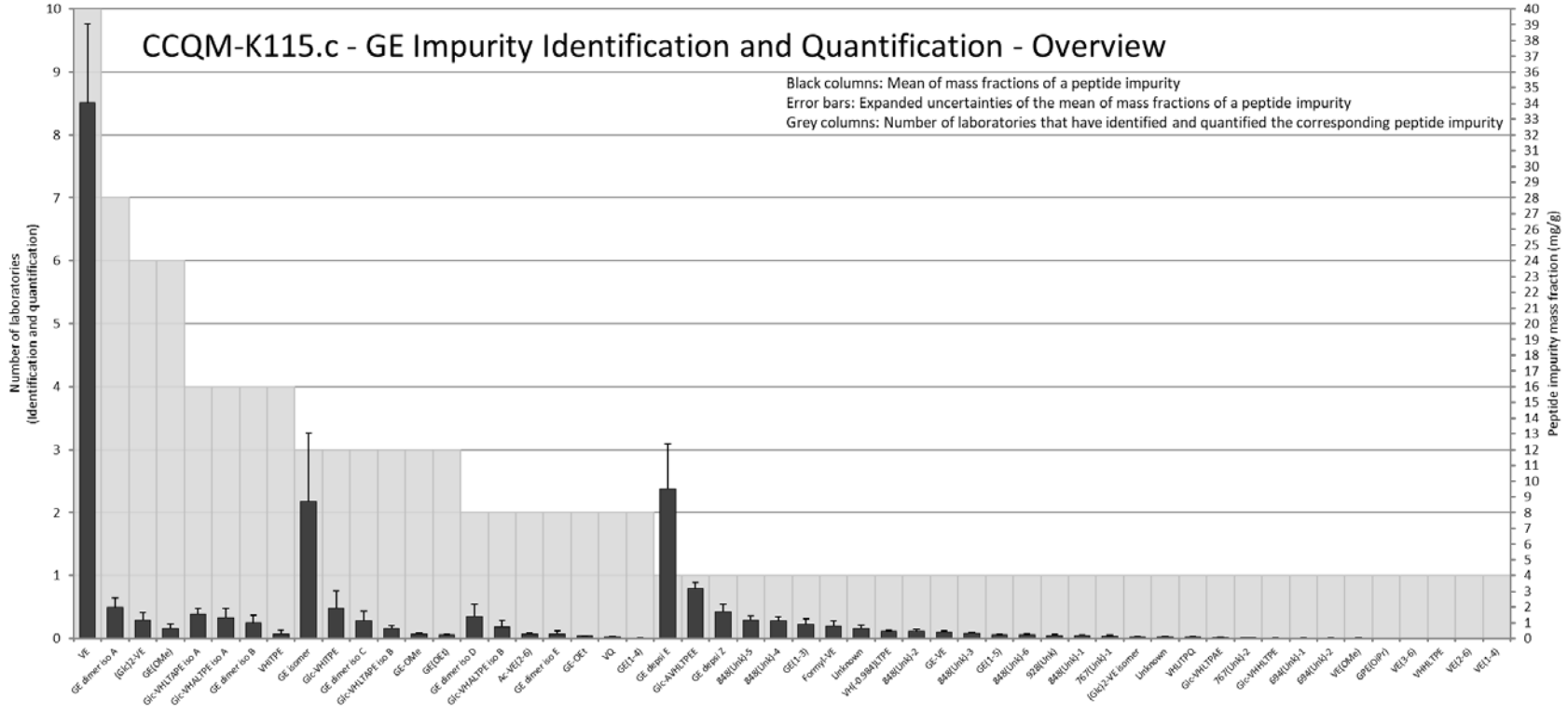


Figure 4: GE impurity identification and quantification - Overview

REFERENCE VALUES (RVS) FOR CCQM-P55.2.C

It was agreed by the CCQM-K115.c and CCQM-P55.2.c participants that the comparison coordinator should establish an individual reference value for the mass fraction of the peptide related impurities (PepImp) present in the comparison material and assign an overall reference value for the mass fraction of GE. The key comparison reference values (KCRVs) of the parallel CCQM-K115.c have been adopted as reference values (RVs) of CCQM-P55.2.c. The approaches to establish the KCRVs for GE and its impurity are described in detail in the Final Report on CCQM-K115.c [24].

Impurity Profile and Reference Value (RV) for Mass Fraction of Peptide Related Impurities in CCQM-P55.2.c

The reference value ($RV_{PepImp} = KCRV_{PepImp}$) for the mass fraction of peptide impurities is based on the assumption that all results of the parallel CCQM-K115.c key comparison are directly taken for the calculation of the RV_{PepImp}. The RV_{PepImp} and the corresponding standard uncertainty ($u(RV_{PepImp})$) was established based on the DerSimonian-Laird variance-weighted mean (DSL) [25-26]. The DSL-mean takes into account the uncertainties while introducing sufficient excess variance (λ) to allow for their observed dispersion. The DSL approach to obtain the RV_{PepImp} has been accepted by all participating NMIs/DIs as none of the results could be excluded for technical shortcomings.
Figure 5 shows the participant results with their reported standard uncertainties plotted against the RV_{PepImp} of 45.4 mg/g for peptide impurities in CCQM-K115.c (solid line) and its corresponding standard uncertainty of 4.2 mg/g ($k = 1$). The excess variance is derived from the dark uncertainty (τ) that of 8.4 mg/g ($\tau^2 = \lambda$). A corresponding expanded uncertainty of 9.5 mg/g ($k = 2.26$) at a confidence level of about 95 % was calculated.

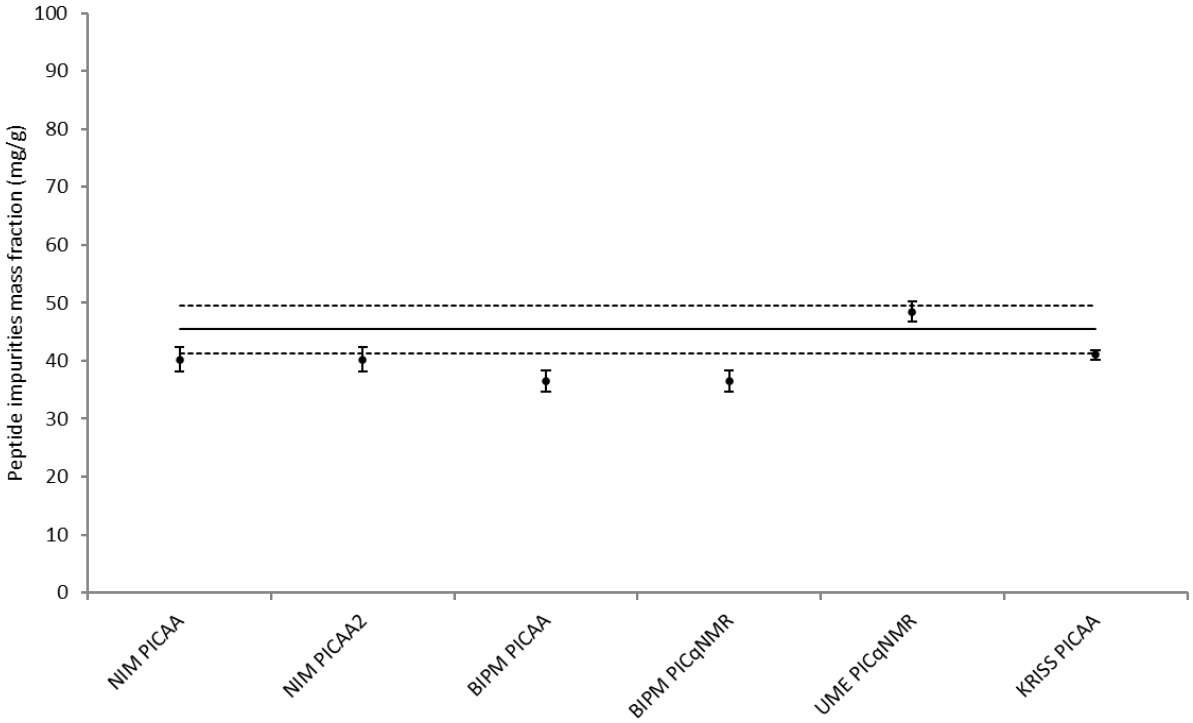


Figure 5: Estimates of total related peptide impurities in CCQM-P55.2.c plotted with their reported standard uncertainties (± u_c, k = 1). The RV_{PepImp} (solid line) is 45.4 mg/g. Dashed lines show the $u(RV_{PepImp})$ (k = 1) of the RV_{PepImp}.

The degree of equivalence of a participant's result with the RV_{PepImp} (D_i) is given by:

$$D_i = w_i - RV_{PepImp}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as [27]:

$$U_{95\,\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + \lambda - u\left(RV_{PepImp}\right)^2}$$

Figure 6 indicates the degree of equivalence (D_i) of each key comparison participant's result with the RV_{PepImp} for related peptide impurities. The corresponding values are listed in Table 4.

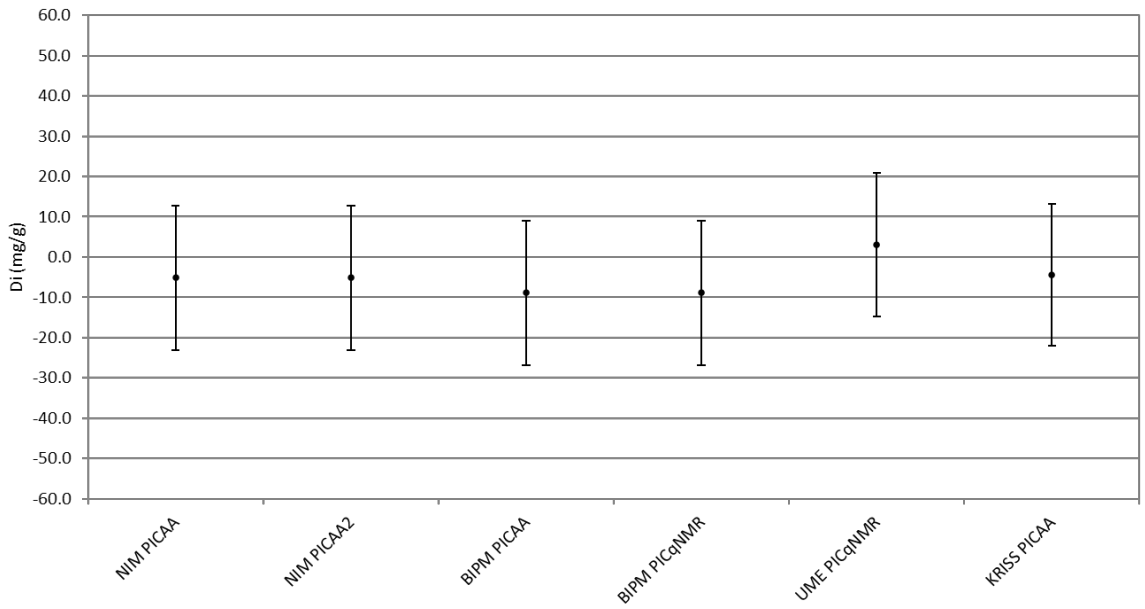


Figure 6: Degree of equivalence for the CCQM-P55.2.c with the RV_{PepImp} for total related peptide impurities for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 4: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for CCQM-P55.2.c with the RV_{PepImp} for total related peptide impurities

	D_i	$U(D_i)$
NIM PICAA	-5.1	18.0
NIM PICAA2	-5.1	18.0
BIPM PICAA	-8.9	17.9
BIPM PICqNMR	-8.9	17.9
UME PICqNMR	3.1	17.8
KRISS PICAA	-4.4	17.6

Reference Value (RV) for the Mass Fraction of GE in CCQM-P55.2.c

The reference value (RV_{GE} = $KCRV_{GE}$) for the mass fraction of GE is based on the assumption that all results of the parallel CCQM-K115.c key comparison are directly taken for the calculation of the RV_{GE}. The RV_{GE} and the corresponding standard uncertainty ($u(RV_{GE})$) was established based on the DerSimonian-Laird variance-weighted mean (DSL) [25-26]. The DSL-mean takes into account the uncertainties while introducing sufficient excess variance (λ) to allow for their observed dispersion. The DSL approach to obtain the $KCRV_{GE}$ has been accepted by all participating NMIs/DIs as none of the results could be excluded for technical shortcomings.
Figure 7 shows the participant results with their reported standard uncertainties plotted against the RV_{GE} of 628 mg/g for GE in CCQM-K115.c (solid line) and its corresponding standard uncertainty of 12 mg/g (k = 1). The excess variance is derived from the dark uncertainty (τ) that of 34.9 mg/g ($\tau^2 = \lambda$). A corresponding expanded uncertainty of 27 mg/g (k = 2.26) at a confidence level of about 95 % was calculated.

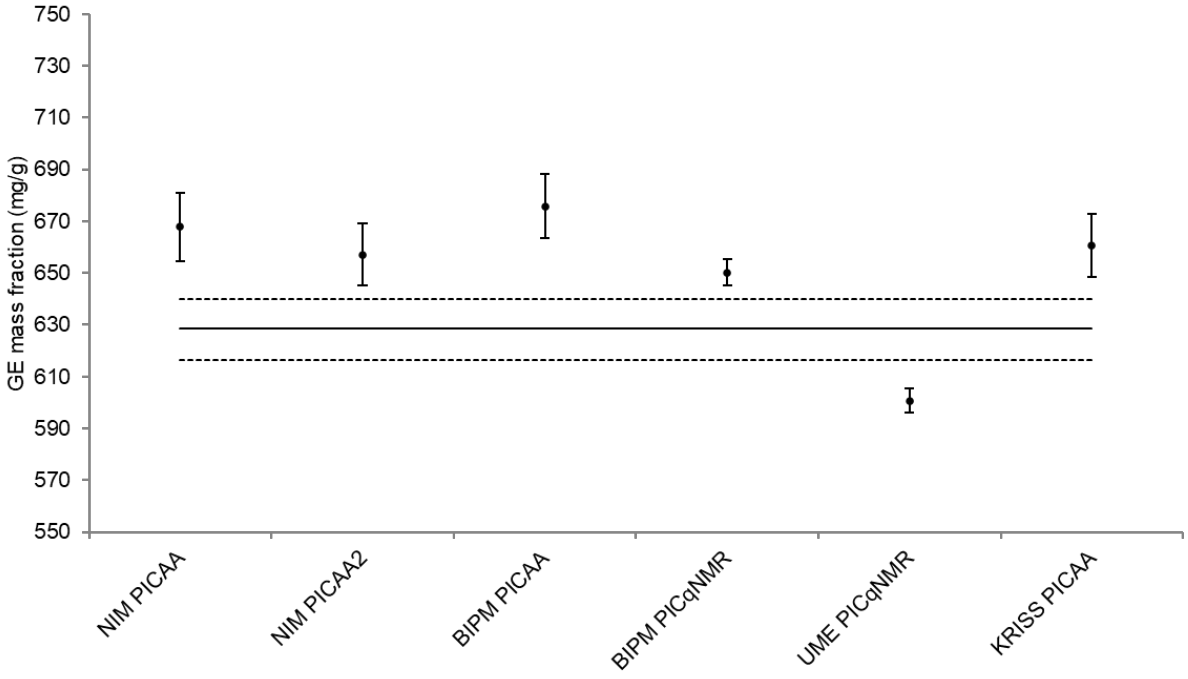


Figure 7: Mass fraction estimates by participants for GE in CCQM-P55.2.c with their reported combined standard uncertainties ($\pm u_c$, k = 1). The RV_{GE} for CCQM-K115.c (solid line) is 628 mg/g. The calculated combined standard uncertainty of the RV_{GE} is ±12 mg/g. Dashed lines show the $u(RV_{GE})$ (k = 1) of the RV_{GE}.

The degree of equivalence of a participant's result with the RV_{GE} (D_i) is given by:

$$D_i = w_i - RV_{GE}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as [27]:

$$U_{95\,\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + \lambda - u(RV_{GE})^2}$$

Figure 8 indicates the degree of equivalence (D_i) of each key comparison participant's result with the RV_{GE} for GE. The corresponding values are listed in Table 5.

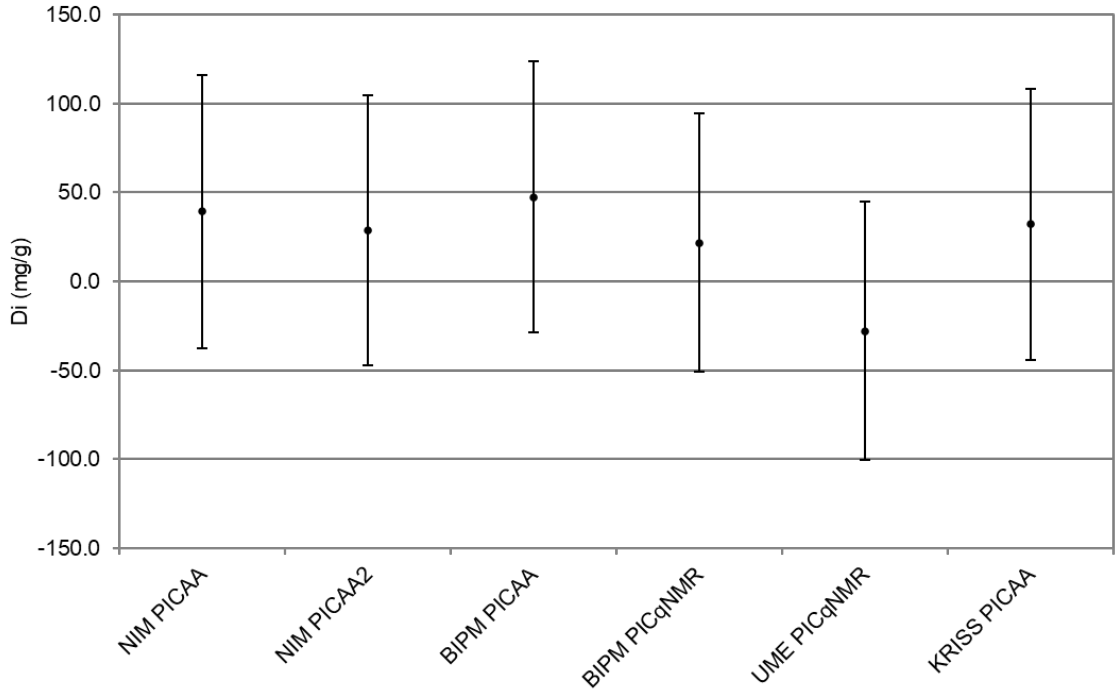


Figure 8: Degree of equivalence for the CCQM-P55.2.c with the RV_{GE} for GE for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 5: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for CCQM-P55.2.c with the RV_{GE} for GE

	D_i	$U(D_i)$
NIM PICAA	39.3	76.6
NIM PICAA2	28.7	75.9
BIPM PICAA	47.4	76.2
BIPM PICqNMR	21.8	72.8
UME PICqNMR	-27.7	72.7
KRISS PICAA	32.2	76.1

CONCLUSIONS

GE was selected to be representative of chemically synthesized linear peptides of known sequence, without cross-links, up to 5 kDa and modification (mono-glycation). It was anticipated to provide an analytical measurement challenge representative for the value-assignment of compounds of broadly similar structural characteristics.

The majority of participants used a PICAA approach as the amount of material that has been provided to each participant (25 mg) is insufficient to perform a full mass balance based characterization of the material by a participating laboratory. The coordinators, both the BIPM and the NIM, were the laboratories to use the mass balance approach as they had more material available.

It was decided to propose KCRVs for both the GE mass fraction and the mass fraction of the peptide related impurities as indispensable contributor regardless of the use of PICAA, mass balance or any other approach to determine the GE purity. This allows participants to demonstrate the efficacy of their implementation of the approaches used to determine the GE mass fraction. In particular, it allows participants to demonstrate the efficacy of their implementation of peptide related impurity identification and quantification.

More detailed studies on the identification/quantification of peptide related impurities revealed that the integrity of the impurity profile of the related peptide impurities obtained by the participant is crucial for the impact on accuracy of the GE mass fraction assignment.

The key comparison reference values (KCRVs) of CCQM K115.c have been adopted as reference values (RVs) of CCQM-P55.2.c. The approaches to establish the KCRVs for GE and its impurity are described in detail in the Final Report on CCQM-K115.c [24].

GE related peptide impurity mass fraction results submitted by all NMIs/DIs of the parallel CCQM-K115.c key comparison are taken directly into account for the calculation of the RV_{PepImp}. The approach selected to obtain a $KCRV_{PepImp}$ is based on random-effects meta-analysis (DerSimonian-Laird (DSL) variance-weighted mean). The DSL-mean takes into account the uncertainties of the results while introducing sufficient excess variance to allow for their observed dispersion resulting in a larger expanded uncertainty $U(RV_{PepImp})$. Consequently, the RV_{PepImp} of

45.4 mg/g is associated with a relatively large corresponding expanded uncertainty of ± 9.5 mg/g (k = 2.26) providing a more realistic basis of evaluation for the capabilities of the participants to identify/quantify peptide related impurities. All GE related peptide impurity mass fraction results for CCQM-P55.2.c are in agreement with the $KCRV_{PepImp}$. Inspection of the degree of equivalence plots for the mass fraction of peptide impurities and additional information obtained from the peptide related impurity profile indicates that in many cases the major related peptide impurities have been identified and quantified with the exception of the GE depsipeptide impurity.

GE mass fraction results submitted by all NMIs/DIs of the parallel CCQM-K115.c key comparison are taken directly into account for the calculation of the RV_{GE}. The approach selected to obtain a RV_{GE} is also based on random-effects meta-analysis (DSL) as the DSL-mean takes into account the uncertainties of the results while introducing sufficient excess variance to allow for their observed dispersion resulting in a larger expanded uncertainty $U(RV_{GE})$. The RV_{GE} for CCQM-K115.c is 628 mg/g with a corresponding expanded uncertainty of the RV_{OXT} of ± 27 mg/g (k = 2.26). Inspection of the degree of equivalence plots for CCQM-P55.2.c for the mass fraction of GE shows that all results agree with the reference value.

The GE material is not sufficiently pure and the corresponding expanded uncertainty is too large to serve as a calibrator to directly support a comparison on the HbA1c quantification in biological samples by IDMS.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

The pilot study CCQM-P55.2.c cannot be used to support CMC claims as the pilot study has its parallel key comparison CCQM-K115.c. However, successful participation in the CCQM-K115.c comparison will support CMCs for:

- chemically synthesized peptides of known sequence, without cross-links, up to 5 kDa and modification (mono-glycation). Additional evidence is required to support claims related to peptides that contain more than 5 kDa, or have been produced using a recombinant process;
- pure peptide primary reference materials value assigned for the mass fraction of the main component peptide within the material;
- methods for the value assignment of the mass fraction of the main component peptide within the material;
- the identification and quantification of minor component peptide impurities within the material.

In addition, the CCQM-K115.c key comparison will support traceability statements of CMCs for peptide and protein quantification which are dependent on pure peptide reference materials or methods for their value assignment for peptides meeting the above criteria.

Glycated hexapeptide of HbA1c (glcVHLTPE or GE) has been proposed as the comparison material, since:

- it will allow the generic capabilities listed above to be demonstrated for modified (mono-glycated) peptides without cross-links and up to 5 kDa molecular mass [1];
- it can be obtained in sufficiently large quantities required for the comparison;
- it will directly support NMI/DI services and certified reference materials currently being provided by NMIs/DIs [28];
- Hemoglobin A1c (HbA1c) is an important analyte for which reference methods have been developed in laboratory medicine [29-34] where GE is the signature peptide for the quantification of HbA1c.

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